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Full Length Research Paper

Effect of varying relative humidity on the rancidity of cashew (*Anacardium occidentale* L.) kernel oil by lipolytic organisms

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Post harvest deterioration by microbes due to improper storage condition is considered to be the major cause of spoilage and rancidity of most oil-bearing seeds like cashew nuts through lipolytic action of lipase enzyme. Roasted cashew nuts were subjected to four different storage conditions with different relative humidity of 30, 70, 80 and 90%, respectively for a period of twelve days. Oil extracted from cashew kernel at each storage condition was examined for rancidity. Eight fungal species and three bacteria were obtained and identified as *Aspergillus niger*, *Rhizopus* sp., *Penicillium* sp., *Trichoderma* sp., *Botryodiplodia* sp., *Fusarium* sp., *Aspergillus flavus* and *Aspergillus ochraceous* while the bacteria isolates were identified as *Bacillus subtilis*, *Bacillus licheniformis* and *Staphylococcus* sp., respectively. Rancidity of extracted cashew oil was observed with cashew kernel stored at 70, 80 and 90% of relative humidity with increase in time while no level of rancidity was detected at 30% of relative humidity judging by the peroxide value, free fatty acid value, specific gravity and acid value of the oil. Isolates with the highest frequency of occurrence were found to be *A. niger* and *Rhizopus* sp. for fungal and *B. subtilis* and *B. licheniformis* for the bacterial isolates. These four isolates were screened for conditions for optimal growth and lipolytic activities in a chemically defined medium. Result of the assay shows that lipase enzyme produced by *A. niger* had the highest lipolytic activity at 37°C.

Key words: Cashew kernel, storage conditions, rancidity, lipolytic.

INTRODUCTION

Cashew nut kernel oil is an important oil that is extracted from the kernel of cashew nut (*Anacardium occidentale* L.). The oil builds up inside the seed as a nutrient reserve and is stored in them as fine droplets. The cashew nut consists of an outer shell epicarp, which is greenish to pinkish brown colour depending on its degree of dryness. Within the outer shell, there is a honey combed structure (mesocarp) in the cells which is secreted as a natural resin, known commercially as cashew nut shell liquid (CNSL). Within the mesocarp, there is an inner shell endocarp which is hard and brittle and protects the kernel from the natural resin (Russell, 1969). There is equally a covering of a thin membrane on the kernel known as the testa which contains anti-oxidant that protects the kernel from penetration by atmospheric oxygen.

Today the crop is extensively cultivated for its nuts, which could be roasted, packaged and marketed or processed into vegetable oil (cashew nut kernel oil) (Adebajo and Diyaolu, 2003). Cashew kernel oil is a good source of vegetable oil which has been recognized and thus has found great use in domestic cooking, pharmaceuticals and in industries for the production of soap and margarine (Ojeh, 1985).

Unfortunately, cashew kernel from which the oil is extracted is very susceptible to deterioration and spoilage. This is due to microbial attack as a result of improper storage. Cashew nut kernel is hygroscopic by its nature and if the critical water content of stored cashew kernel is exceeded due to poor storage, it promotes hydrolytic/ enzymatic fat cleavage. Microbial attack leads to hydrolysis of the oil therein and hence the formation of free fatty acids, the accumulation of which is responsible for the rancid flavour/odour of deteriorated cashew kernel oil (Kirk and Sawyer, 1991). In fact, the quality and market

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value of cashew kernel oil can be determined, among other criteria by the level of free fatty acids it contains.

Because of the importance of microorganisms in the spoilage, there have been investigations into organisms responsible. In one of the investigations on the mycology and spoilage of retail cashew nut (Adebayo and Diyaolu, 2003), 14 fungi belonging to 5 genera were recovered at varying level from the cashew nuts.

All earlier investigations were concentrated on the mycology of the nuts without reference to the bacteria involved at the different storage conditions and the fate of the oil therein. The objective of this study is therefore aimed at studying the effect that varying relative humidity will have on the deterioration of cashew nut kernel under storage, the rancidity of the oil therein and the characterization of the lipolytic activities in the microbial isolates *in vivo*.

MATERIALS AND METHODS

Collection of samples

Good grade cashew nut of the current season was obtained from the storage unit of Cocoa Research Institute of Nigeria. The nuts were carefully selected to ensure that infected ones were not included.

Removal of cashew kernel and storage

The cashew kernels were isolated using a simple cutter knife. This was used to slit each nut open and a pointed knife employed to remove the kernel immediately from the shell to minimize contamination with the cashew nut shell liquid (CNSL). The kernel was then subjected to roasting at 80°C for one hour to remove the testa.

Four relative humidity levels of 30, 70, 80 and 90% of storage were chosen for investigation and these were created using different non-toxic desiccant inside a clean sterilized dessicator prepared according to the method in the Hawaian Seed Manual (www.hawaii.edu/scb/doc/science/seed/seedmanual.html.).

Weighted amounts (120 g) of the roasted cashew kernel on sterile Petri-dishes were aseptically placed in each of the dessicators (with perforated holes), sealed with Vaseline to ensure it was air tight and incubated at room temperature (27 ± 2°C). Samples were taken after every three days from each of the storage for analysis for a period of 12 days (3, 6, 9, and 12 days).

Isolation of microorganisms from the samples

Considering the design of the experimental set up of 0, 3, 6, 9 and 12 days at different relative humidities of storage, samples were randomly taken from the different dessicators, weighed and surface sterilized with 0.1% of mercury chloride solution ($HgCl_2$) for about 2 min. This was followed by three rinses with sterile distilled water. 10 g of the nut was pulverized by grinding it with sterile mortar and pestle. The initial dilution was prepared by adding 10 g of the pulverized sample to 90 ml of distilled water. Decimal dilutions and pour-plating were prepared down to 10^{-3} using the serial dilution method of Meynell and Meynell (1970) and Harrigan and MacCance (1974). Before pouring plates, chloramphenicol was added at a concentration of 50 mg/l to potato dextrose agar (PDA) to inhibit the growth of bacteria while nystatin was equally added at a concentration of 50 mg/l to nutrient agar to inhibit the growth of fungi. Solidified Petri-dishes were incubated upright at room temperature for 24 - 48 h respectively. The colonies observed were counted and sub-cultured for identification.

Identification of Isolates

All the isolates were identified using microscopic, biochemical and physiological characteristics and by reference to identification manuals (Sneath, 1986).

Effect of different storage conditions on the physical state of the samples

At different time intervals (0, 3, 6, 9 and 12 days) samples of the kernel were picked from each of the dessicators to determine the level of crispness. This was done by bending the kernel in between the finger. The crispy ones break easily while the non-crispy ones were wet (Adebajo and Diyaolu, 2003).

Extraction of cashew kernel oil

This employed the use of Soxhlet apparatus. The extraction was carried out according to the procedure of AOAC (1990). After every 3 days, in each dessicator, sample of the cashew kernel from the different storage were blended, 25 g of the grinded kernel was packed in a filter paper and introduced into porous thimble. In this case, n-hexane was used as the extracting solvent and this was effected for 6 - 7 h. At the end of the extraction, the mixture was concentrated by distilling off the solvent, the oil was desolventized and concentrated.

Determination of peroxide value (PV) of cashew kernel oil.

Peroxide value in the oil was determined using the standard method of American Oil Chemist Society AOCS (1978) using glacial acetic acid, chloroform: 0.01 M sodium thiosulphate and potassium iodide (KI) 10% as the major solvents in 1.0 g of oil sample.

Determination of refractive index of extracted cashew oil

This was done using the Abbe refractometer. This equipment was first standardized with water, to a refractive index of 1.33. Thereafter, the meter was cleaned with a cotton wool and a drop of the oil placed on it and the refractive index determined for each sample.

Determination of specific gravity of extracted oil

This was done by using the density bottle. A known specific volume of cashew kernel oil extracted was poured into the density bottle and the weight was measured. Water at the same volume was also poured into the density bottle and was weighed. The specific gravity was then determined using (AOAC 1990).

Determination of acid value (AV) and free fatty acid (FFA) of cashew kernel oil

This was also determined by the standard methods of the American Oil Chemists Society (1976) using diethyl ether, ethanol, phenolphthalein and sodium hydroxide as the reagents.

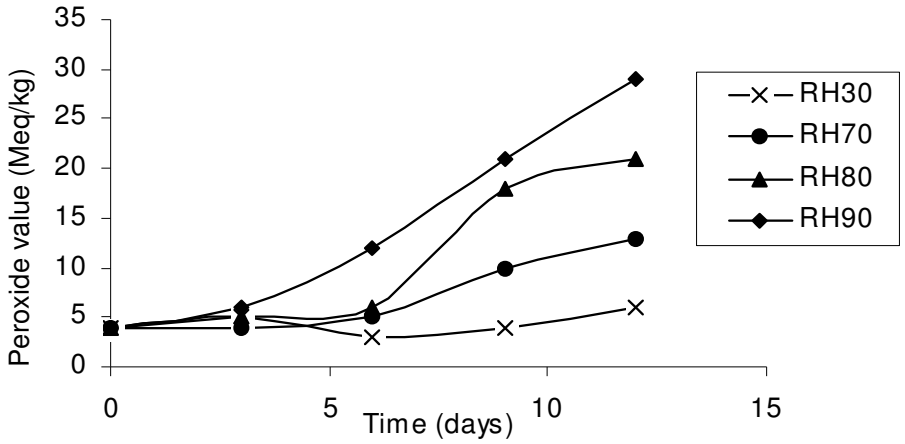

Figure 1. Peroxide value of cashew oil obtained after storage of the cashew kernel at different relative humidities for 12 days.

Screening for lipase production by the isolates

Two frequently occurring fungal isolates-*Aspergillus niger* and *Rhizopus* sp. were grown in a chemically-defined medium (Baillageon et al., 1989). The basal medium contained 5% peptone, 0.1% $MgSO_4$ and 0.1% $NaNO_3$. Sterilization was carried out in an autoclave at 121°C for 15 min. Sterile (0.5%, v/v) glycerol was added as a carbon source. The medium was aseptically dispensed into sterile MacCatney bottles, inoculated with the spores of the isolates and incubated at 32°C for 5 days.

After obtaining the maximum growth, the cultures were filtered through Whatman No. 1 filter paper to remove the mycelia. A clear filtrate was obtained containing the crude enzyme that was used to assay for lipase enzyme.

Also, two bacterial isolates that frequently occurred were *Bacillus subtilis* and *Bacillus licheniformis* and these were cultured in a chemically-defined medium of the following composition (gram per litre of distilled water) (Fang and Demain, 1989); L-aspartic acid (2.0), L-arginine (1.0), DL-methionine (0.4), glycine (0.4), K_2HPO_4 (6.0), KH_2PO_4 (2.0), NH_4CL (1.0), NH_4NO_3 (0.2), Na_2SO_4 (0.2), $MgSO_4$. $7H_2O$ (0.004), $MnSO_4$. $4H_2O$ (0.002), $FeSO_4.7H_2O$ (0.002) and $CaCl_2$ (0.001). Sterilization was done in an autoclave at 121°C for 15 min. Sterile glycerol serves as the carbon source and was aseptically added after separate sterilization and the pH adjusted to 6.8. The medium was then aseptically dispensed into sterile MacCatney bottles, inoculated with organisms and incubated at 32°C for 24 h.

Lipase enzyme assay

Lipolytic activity of the filtrate of both bacteria and fungi was determined using the modified method of Parry et al. (1966) using as substrate a 10% olive oil-gum arabic solution emulsified by sonication for 2 min according to Linfield et al. (1985). 1 millilitre of cell-free fermentation broth prepared by centrifugation was added to 5 ml of 10% olive oil gum arabic solution emulsion and incubated at room temperature for 1 h with rapid stirring. 1.0 ml of 95% ethanol was added to stop the reaction and the free fatty acid produced was quantified by titration to pH 9.5 using Hanna pH meter with 0.1N sodium hydroxide (NaOH) (Baillargeon, et al., 1989). Blanks with 1.0 ml of uninoculated and unincubated broth served as the control. A unit of lipase activity is defined as the amount of NaOH used in the titration to bring the reaction mixture to pH of 9.5 under the defined assay condition (Young and Wood, 1977).

Effect of temperature on enzyme activity

This was investigated by subjecting the reaction mixture to different temperature ranges of 4, 10, 30, 37 and 45°C. Total free fatty acid liberated was determined after 5 days of incubation.

RESULTS

A total number of eight fungal isolates were obtained from the various treatments of the cashew nuts. They were identified as *A. niger, A. flavus, Penicillium* sp., *Botryodiplodia* sp., *Rhizopus* sp., *Fusarium compactum, Trichoderma* sp. and *A. ochraeous* while the bacteria isolates were identified as *B. subtilis, B. licheniformis* and *Staphylococcus* sp. The succession of growth of the various fungal isolates in the various treatments differed as some fungi were eliminated with the treatment. However two fungi isolates *Rhizopus sp* and *A. niger* were predominantly encountered in all treatments but their number were greatly affected as depicted in the fungal count.

The influence of the different relative humidities on the physical state of cashew nut at the different time intervals was equally evaluated. Treatment at 30% relative humidities showed the kernels to be very hard and crispy. At 70, 80 and 90% relative humidities, the nuts were a bit crispy on the 3rd day of examination and these decreased with time. On the 9th and 12th day, the nuts stored at 90% relative humidity had already grown mouldy and wet.

Figure 1 show the peroxide value obtained from the experiment. The results reveal that up till 80% relative humidity, the peroxide value stabilized for the first three days beyond which gradual increase in observed values

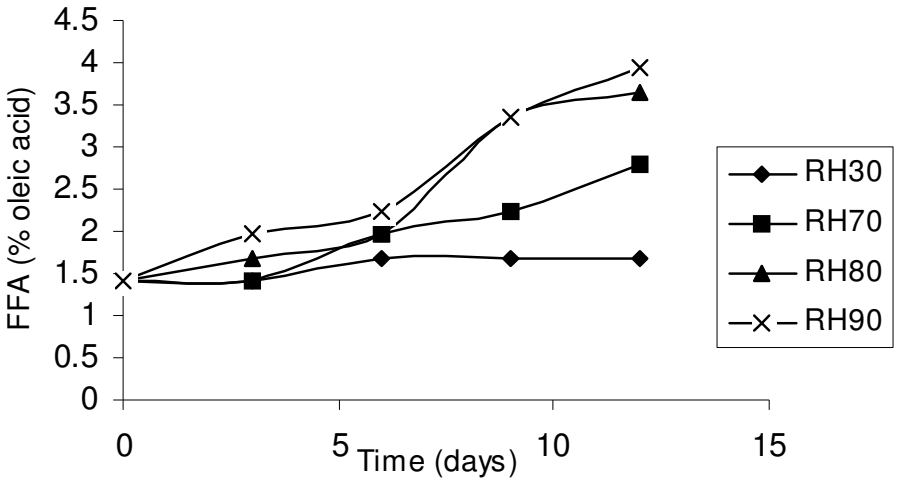

Figure 2. % free fatty acid of cashew oil obtained after storage of the cashew kernel oil at different relative humidities for 12 days.

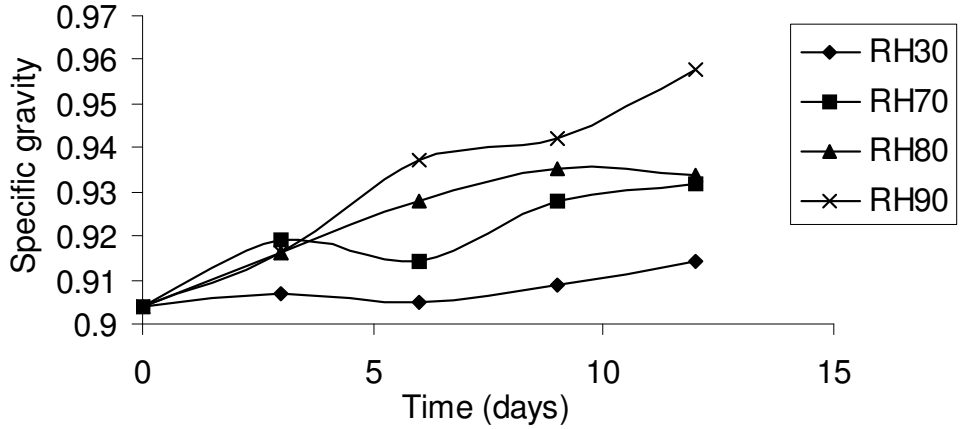

Figure 3. Specific gravity of cashew oil obtained after storage of the cashew kernel at different relative humidities for 12 days.

were noted. At 80% relative humidity, the increase after the first 6 days was sharp until a peak value of 21.0 Meq/kg was obtained on the 12th day. Observed values at 90% relative humidity was also sharper with a higher peak of 29.0 Meq/kg observed on the 12th day.

The values obtained for percentage free fatty acid are shown in Figure 2. The results revealed that high values were obtained at relative humidities of 90, 80 and 70%. At 30% relative humidity, the free fatty acid values stabilized for the first 3 days beyond which slight increase in observed value was noted from 1.403 to 1.683 (%oleic acid) on the 6th day. This value stabilized up to the 12th day. The increase in free fatty acid value after the first 6 days of storage up to the 9th day at 80% relative humidity was very sharp until a peak value of 3.646 (% oleic acid) was obtained on the 12th day. Observed value at 90% relative humidity was also sharper with a higher peak of 3.927 (% oleic acid) observed on the 12th day.

Figure 3 shows the specific gravity of cashew oil obtained at different relative humidities and different time intervals. From the figure, it was observed that at relative humidity of 30%, the specific gravity values obtained throughout the course of experiment was relatively low and found to increase with time. At relative humidity of 70%, the specific gravity increased sharply after the 6th day of storage until a peak value of 0.932 g was obtained on the 12th day while at 80 and 90% of relative humidity, increase in specific gravity was noticed after the 6th day of storage. There was a sharp increase in value at 90% with a peak value of 0.958 g obtained on the 12th day of investigation.

Figure 4 shows the acid value of cashew nut oil obtained at different relative humidity with respect to different time intervals. At 30 and 70% relative humidities, the acid value stabilized for the first 3 days with a value of 2.085, beyond this point at 30% relative humidity, the

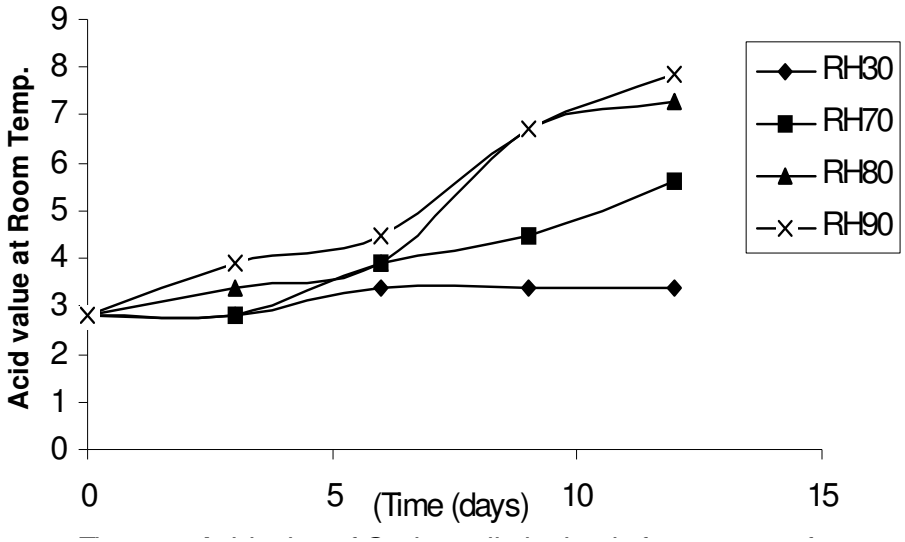

Figure 4. Acid value of cashew oil obtained after storage of the cashew kernel at different relative humidities for 12 days.

Table 1. Lipolytic activity of isolates after 1 h of incubation.

Isolates	Lipolytic activity (units/ml)
Bacillus subtilis	0.1417
Bacillus licheniformis	0.1333
Rhizopus sp.	0.1917
Aspergillus niger	0.2083

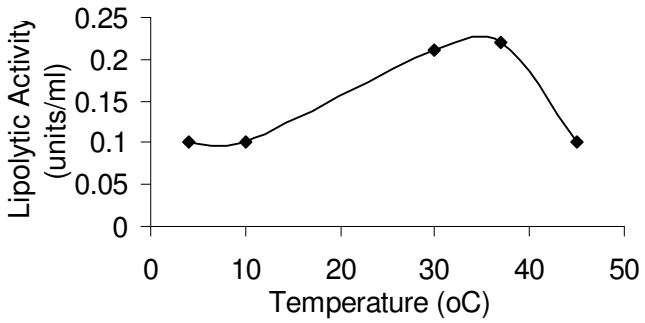

Figure 5. Effect of different temperatures on the lipolytic activity of *A. niger*.

acid value increased slightly to 3.366 on the 6th day and then stabilized at this value up to the 12th day. Sharp increase in value was equally observed at 80 and 90% with a value of 6.732 on the 9th day with a higher peak value of 7.854 obtained on the 12th day at 90% relative humidity.

Based on the frequency of occurrence of the isolates two fungi and two bacterial were screened for lipolytic activity. Table 1 indicates lipolytic activity of the four microbial isolates (*B. subtilis, B. licheniformis, Rhizopus* sp. and *A. niger)* in broth system; *A. niger* produced the highest free fatty acid followed by *Rhizopus* sp., *B. subtillis* and *B. licheniformis*, respectively. *A. niger* the highest producer was then chosen for further studies.

The effect of temperature on the lipolytic activity of *A. niger* was carried out as shown in Figure 5. The broth was incubated at different temperature ranges of 4, 10, 30, 37 and 45°C respectively for a period of five days and the free fatty acids produced were determined. From the result, there was an increase in lipolytic activity as the temperature progressed and the optimum temperature of lipase activity was observed at 37°C. At temperature of 45°C there was a marked decrease in lipolytic activity. Conversely, low temperature ranges were not favourable for lipase enzyme production as observed.

DISCUSSION

Fungi were the predominant organisms isolated from stored cashew nut kernel at different storage relative humidity, with few bacterial isolates. The fungal isolates were identified as *Rhizopus sp, A. niger, A. flavus, A. ochraceous, Botryodiplodia* sp., *Penicillium* sp., *F. compactum* and *Trichoderma* sp., while the bacterial isolates are *B. subtilis, B. licheniformis* and *Staphylococcus* sp. Some group of these fungi has been reported by Adebajo and Diyaolu (2003) as the most predominantly encountered species of fungi from deteriorating cashew nuts. Some of the species, especially *Aspergillus* and *Penicillum* associated with nut are known to have strain that can produce toxic metabolites (Bamburg et al., 1969; Cole and Cox, 1981). Thus they pose a potential hazard to consumers' health.

The most frequently encountered fungi species with the

highest frequency of occurrence were *Rhizopus* (34.9%) and *A. niger* (32.6%). This conforms with the findings of Adam and Moss (1999) that the most important lipolytic mould are species of *A. niger, A. tamari* and *Penicillium* sp. while at higher water activities, species of *Rhizopus* may be implicated. Also, Salunkhe and Desai (1986) observed that storage mould especially *Aspergillus* group develops quickly under high humid conditions, increasing the fatty acid content of oil seeds.

For the bacterial isolates, few were encountered. This finding could probably be due to the fact that cashew nut being a slightly acidic product in terms of the inner content does not support the growth of bacteria.

The circumstantial evidence for the involvement of bacteria in the deterioration of stored cashew nuts is the ability of the spores of bacillus to resist desiccation and this allows their survival in dried products (Adams and Moss, 1999).

The lipolytic activities of *Bacillus sp.* are well known; they have been isolated from oily foods. From the studies conducted by Jonsson and Snygg (1974), *B. licheniformis* was one of the organisms identified with the greatest ability to hydrolyse the fat in mayonnaise and liver paste. Also, Odunfa (1989) observed *B. subtilis* to be strongly lipolytic on palm oil.

Further evidence in support of this is the fact that insufficient heating which will destroy vegetative cells, may not destroy spores of *B. subtilis,* some of which are known to be resistant to 100°C for 15-20 min (Atlas and Bartha, 1981) and may even withstand higher temperatures in a protective medium such as oil and cashew nut.

The moisture content (%) recorded for the cashew nuts during the different storage conditions ranged between 4.0 and 12.0. Storage at 30% relative humidity throughout the period of time shows that the moisture content was relatively constant at 4.0 hence the nuts were hard, crispy and were not predisposed to microbial spoilage.

Relative humidity of 70, 80 and 90% shows an increase of moisture content with time, with peak value of 12.0% observed after 12 days of storage at 90% of relative humidity when the nuts had already grown mouldy. This shows that cashew nut being colloid, are hygroscopic; absorbing moisture from the surrounding atmosphere hence the increase in moisture content above the storage limit observed which consequently predisposed the nut to mouldy growth. This is in agreement with the finding of Pixton (1967) who observed that agricultural products including cashew nut would absorb moisture from the surrounding atmosphere until they are in equilibrium with it.

Solvent extraction method was used in extracting oil from the cashew nut and various parameters were used to determine the level of rancidity of the oil. The peroxide value obtained showed that storage of the nut at relative humidity of 30% did not cause any rancidity of the oil throughout the period of investigation. However, with relative humidity of 70, 80 and 90% after some period of storage, the peroxide value increased above the allowable limit of 10 Meq/kg. It has been reported that a rancid taste and odour often begins to be noticeable when the peroxide value is between 20 and 40 Meq/Kg (Kirk and Sawyer, 1991). At this level, complex chemical changes occur and volatile products are formed that are mainly responsible for the rancidity. Peroxide value (PV) is used for assessing the extent to which spoilage has advanced in oil (Swoboda, 1973).

The acid value (AV) and free fatty acid is a measure to which the glyceride in the oil have been decomposed by lipase action (Kirk and Sawyer, 1991). Storage at 30% of relative humidity did not cause rancidity while at other relative humidities, rancidity was observed after some time. This is due to the fact that at higher relative humidity, traces of moisture could not be ruled out, hence the production of lipase enzyme was favoured. Lipase enzyme hydrolyses esters in oil preferentially at the interface between lipid and water in heterogenous system (Corzo and Revah, 1999).

The specific gravity (S.G) of oil obtained shows that at low relative humidity of 30%, the specific gravity falls within narrow range of 0.904 - 0.914, while at higher relative humidity of 70, 80 and 90%, the value increased with time due to rancidity that had developed. The specific gravity of oil tends to increase when rancidity develops in oil, and this is used in conjunction with other parameters in assessing the purity of oil (Josyln, 1976).

The refractive index of oil is widely used as criterion of identity. The values obtained are within the limit of the oil (1.465) and compares with the results of other workers. Josyln (1976) for example reported the refractive index of coconut oil to be 1.453 while that of walnut oil is 1.477.

Lipolytic activity of the four microbial isolates that were frequently encountered was studied. *B. subtilis, B. licheniformis, Rhizopus sp.* and *A. niger* were monitored using the broth system that was supplemented with olive oil a procedure similar to that of Baillargeon and co-workers (1989). Lipases from non-lactic bacteria such as B. subtilis have been reported to be mainly found in the extracellular medium (Lee and Rhee, 1993; Lesuisse et al., 1993). Among the four isolates, *A. niger* had the highest lipolytic activity followed in descending order by *Rhizopus* sp., *B. subtiis* and *B. licheniformis*.

Based on this, the optimum condition for lipolytic activity of A. *niger* was further investigated. Considering temperature as a parameter for lipolytic activity, the highest fatty acid was released by *A. niger* lipase at 37°C. This result is in conformity with the findings of Chander et al. (1977) and Eitenmiller et al. (1970) in *Penicillium roqueforti* and *Penicillium chrysogenum* lipases, respectively. Above this temperature, there was a marked decrease in lipolytic activity showing the enzymes to be very sensitive to high temperature, which could lead to denaturation. Most reports including this one show that lipase activity of *A. niger* is greatest at 37°C; since cashew kernel is normally stored at room temperature, the

lipase activity of most lipolytic organisms like *A. niger* will be enhanced if not stored at the suitable relative humidity.

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